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Abstract

The purpose of this review is to summarize the present knowledge on the interplay among the cholinergic system, Extracellular signal-Regulated Kinase (ERK) and Mammalian Target of Rapamycin (mTOR) pathways in the development of short and long term memories during the acquisition and recall of the step-down inhibitory avoidance in the hippocampus. The step-down inhibitory avoidance is a form of associative learning that is acquired in a relatively simple one-trial test through several sensorial inputs. Inhibitory avoidance depends on the integrated activity of hippocampal CA1 and other brain areas. Recall can be performed at different times after acquisition, thus allowing for the study of both short and long term memory. Among the many neurotransmitter systems involved, the cholinergic neurons that originate in the basal forebrain and project to the hippocampus are of crucial importance in inhibitory avoidance processes. Acetylcholine released from cholinergic fibers during acquisition and/or recall of behavioural tasks activates muscarinic and nicotinic acetylcholine receptors and brings about a long-lasting potentiation of the postsynaptic membrane followed by downstream activation of intracellular pathway (ERK, among others) that create conditions favourable for neuronal plasticity. ERK appears to be salient not only in long term memory, but also in the molecular mechanisms underlying short term memory formation in the hippocampus. Since ERK can function as a biochemical coincidence detector in response to extracellular signals in neurons, the activation of ERK-dependent downstream effectors is determined, in part, by the duration of ERK phosphorylation itself. Long term memories require protein synthesis, that in the synapto-dendritic compartment represents a direct mechanism that can produce rapid changes in protein content in response to synaptic activity. mTOR in the brain regulates protein translation in response to neuronal activity, thereby modulating synaptic plasticity and long term memory formation. Some studies demonstrate a complex interplay among the cholinergic system, ERK and mTOR. It has been shown that co-activation of muscarinic acetylcholine receptors and β-adrenergic receptors facilitates the conversion of short term to long term synaptic plasticity through an ERK- and mTOR-dependent mechanism which requires translation initiation. It seems therefore that the complex interplay among the cholinergic system, ERK and mTOR is crucial in the development of new inhibitory avoidance memories in the hippocampus.

Keywords: Memory, hippocampus, acetylcholine, ERK, mTOR, Inhibitory avoidance

Abbreviations

4E-BPs: 4E binding proteins
ACh: Acetylcholine
AD: Alzheimer's disease
APP: Amyloid precursor protein
CaMKII: Ca2+/calmodulin-dependent protein kinase II
CREB: cAMP response element-binding protein
DG: Dentate gyrus
eEF1A: eukaryotic Elongation Factor 1A
eEF2: eukaryotic Elongation Factor 2
ERK: Extracellular signal-regulated kinase
GABA: gamma-aminobutyric acid
GPCRs: G protein–coupled receptors
IA: Inhibitory avoidance
ICV: intracerebroventricular
IP: intraperitoneal
JNK: c-Jun N-terminal kinase
LTM: Long Term Memory
LTP: Long term potentiation
mAChRs: Muscarinic acetylcholine receptors
M1,...,M5: Muscarinic receptor 1,...,5
MAP: Microtubule-associated proteins
MAPK: Mitogen activated protein kinase
MEK: Mitogen-activated protein kinase kinase
mTOR: Mammalian Target of Rapamycin
mTORC1: mTOR Complex1
nAChRs: Nicotinic acetylcholine receptors
NBM: nucleus basalis magnocellularis
NMDA: N-methyl-D-aspartate
p38MAPK: p38 Mitogen Activated Protein Kinase
p70S6K: p70 ribosomal subunit S6 Kinase
PKA: Protein Kinase A
PKC: Protein Kinase C
STM: Short Term Memory
TgCRND8: Transgenic Centre for Research in Neurodegenerative Diseases 8
wt: wild type

1. Introduction

As St. Augustine wrote in the "Confessions" in the IVth Century a.d. "*And I come to the fields and spacious palaces of my memory, where are the treasures of innumerable images, brought into it from things of all sorts perceived by the senses. ... Nor yet do the things themselves enter in; only the images of the things perceived are there in readiness, for thought to recall. Which images, how they are formed, who can tell, though it doth plainly appear by which sense each hath been brought in and stored up?*." (St.Augustine, 398). The purpose of this review is to try to answer to the question that already fascinated St. Augustine on how memories are formed, by summarizing some of the present knowledge on the mechanisms that underlie memory development in our brain.

The formation of memories is the result of cellular and molecular mechanisms activated in different structures of the brain. The ability of an animal to adapt its behaviour in response to environmental stimuli depends on the structural and functional plasticity of several brain regions. Therefore, it is of the utmost importance to understand how and where in the brain experiences are encoded into lasting memories.

A single learning experience starts a cascade of events, which can lead to different forms of memory: short-term memory (STM) that lasts minutes to hours and long term memory (LTM) that lasts days, weeks, and even a lifetime (McGaugh, 1966). A major question of memory neurobiology is whether these two forms are related or independent phenomena. Some cellular mechanisms that underlie the development of STM overlap with those of LTM, but other mechanisms are independent (Izquierdo et al., 1998a; Izquierdo, Medina, Vianna, Izquierdo, & Barros, 1999; Izquierdo et al., 2002). A unique characteristic of LTM is the need for a consolidation period during which synaptic, structural, and functional modifications occur (Igaz, Vianna, Medina, & Izquierdo, 2002). The most important is protein synthesis on which LTM, but not STM, depends (Davis & Squire, 1984; Freeman, Rose, & Scholey, 1995; Tiunova, Anokhin, Rose, & Mileusnic, 1996; Bourtchouladze et al., 1998; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999; Quevedo et al., 1999).

Memory is not a unitary function. Memory depends on the integrated activity of various brain structures and neurotransmitter systems and involves multiple receptors, postsynaptic mechanisms, and signal transduction pathways (Izquierdo et al., 1998a). Among the various brain structures implicated in memory formation, the CA1 region of the hippocampus plays a major role in memory encoding (Squire, 1992; Hasselmo, Wyble, & Wallenstein, 1996; Vinogradova, 2001; Eichenbaum, 2001; Lisman & Grace, 2005).

Step-down inhibitory avoidance memory

The step-down inhibitory avoidance (IA) is a form of associative learning that is acquired in one trial through several sensory inputs. IA memory depends on the integrated activity of CA1, entorhinal and posterior parietal cortex, and is modulated by the amygdala and by the basal forebrain cholinergic neurons of the medial septum and indirectly by stress hormones (Izquierdo, 1989; Izquierdo & Medina, 1997; Cammarota, Bevilaqua, Medina, & Izquierdo, 2007). The step-down IA is a widely used task in memory studies (McGaugh, 1966; Gold, 1986; McGaugh & Izquierdo, 2000; Izquierdo et al., 2007) and relies upon the natural tendency of an animal to explore a novel environment. In the IA acquisition task, the animal is placed on an elevated platform by one wall of an arena, steps down to explore the arena and learns to associate exploration with a punishment (a foot shock delivered through the floor grid). On a subsequent exposure to the same environment (recall task), the animal increases the latency to step down onto the floor grid, or avoids stepping on the grid. The natural exploratory behaviour is repressed after the punishment is given, without affecting the exploratory behaviour while on the safe, non-aversive part of the training apparatus. IA is an emotionally-arousing paradigm (Izquierdo et al., 1997; Maren, 2001), that involves:

i) an explicit, associative component to the context,

ii) an operant-like conditioning component to the shock, since the animal may avoid the aversive stimulus (Wilensky, Schafe, & LeDoux, 2000),

iii) a spatial memory component, since the animal remembers the location where the noxious stimulus was given during acquisition (Cammarota, Bevilaqua, Medina, & Izquierdo, 2007).

In the IA, the environment is arranged so that the animal can avoid a painful stimulus; i.e., the "escape" or avoidance is an option available to an animal that could learn and perform it. From an experimental view point, IA is a relatively simple test since it is acquired in a one-trial session. Recall can be performed at different times after acquisition, thus allowing to study both STM (Izquierdo et al., 1998a; Izquierdo et al., 1998b) and LTM mechanisms (Izquierdo et al., 2002).

IA depends upon the activation of the cholinergic system, since its acquisition is impaired by pre-training (Izquierdo et al., 1998b; Giovannini, Bartolini, Bacciottini, Greco, & Blandina, 1999) or post-training peripheral administration of mAChRs antagonists (Table 1) (Izquierdo et al., 1998b; Giovannini, Bartolini, Bacciottini, Greco, & Blandina, 1999; McGaugh & Izquierdo, 2000), and is facilitated by mAChRs agonists (Baratti, Huygens, Mino, Merlo, & Gardella, 1979; Barros, Pereira, Medina, & Izquierdo, 2002).

Short term and long term memory mechanisms: open questions

All types of novel stimuli induce the activation of the forebrain cholinergic system (Pepeu and Giovannini, 2006). In this review we shall examine how the cholinergic system participates in the formation of STM and LTM in CA1 during the acquisition and performance of the step-down inhibitory avoidance task in the rat. A key question that still remains unanswered is whether STM represents a step toward LTM only or the formation of the two memory types reflects separate processes.

According to current hypotheses, STM and LTM formation imply biochemical processes that act in parallel and on different time scales (Izquierdo et al., 1998a; Izquierdo, Medina, Vianna, Izquierdo, & Barros, 1999; Izquierdo et al., 2002). Nevertheless, to better answer to this question, it is necessary to demonstrate that STM can be suppressed without affecting LTM. The pharmacology and molecular bases of IA have been studied by us (Giovannini et al., 2005; Lana et al., 2013) and Izquierdo's group, particularly in the CA1 region (Igaz, Bekinschtein, Izquierdo, & Medina, 2004; Marti, Ramirez, Dos Reis, & Izquierdo, 2004). Moreover, for the reasons mentioned above, unlike multitrial learning tasks, IA offers the possibility to neuroscientists to distinguish the processes involved in STM and LTM by the simple modulation of time parameters after the acquisition task. In particular we shall try to shed light on the complex interplay among the cholinergic system, ERK and mTOR in IA memory formation. Among the several actors downstream of the cholinergic activation implicated in STM and LTM formation, this review will focus particularly on ERK and mTOR since they can modulate both early processes such as phosphorylation of protein substrates, implicated in STM, and later processes like immediate or *de novo* proteosynthesis in neurons, implicated in LTM formation (Davis & Squire, 1984; Freeman, Rose, & Scholey, 1995; Tiunova, Anokhin, Rose, & Mileusnic, 1996; Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Bourtchouladze et al., 1998; Quevedo et al., 1999; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999; Cammarota et al., 2000; Alonso, Viola, Izquierdo, & Medina, 2002; Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007Myskiw et al., 2008).

2. The hippocampal cholinergic system in learning and memory

Among the many neurotransmitter systems, the cholinergic fibres that originate in the basal forebrain and project to the hippocampus are of crucial importance in learning and memory processes (Zola-Morgan & Squire, 1993;Muir, Everitt, & Robbins, 1996;Everitt & Robbins, 1997;Sarter & Bruno, 1997a;Sarter & Bruno, 2000). The hippocampus receives a large cholinergic input (Frotscher & Leranth, 1985) from neurons located in the medial

septum and the vertical limb of the diagonal band of Broca, denominated by Mesulam Cholinergic sector 1 (Ch1) and Cholinergic sector 2 (Ch2) (Mesulam, Mufson, Vainer, & Levey, 1983). These neuronal clusters are parts of the forebrain cholinergic system, formed by an aggregate of discontinuous islands of multipolar cells with extensive dendritic trees. An analysis of the targets of the cholinergic fibers shows that pyramidal cells, granule cells, and non-pyramidal neurons of the hippocampus receive cholinergic input (Frotscher & Leranth, 1985). ACh, released from the cholinergic terminals, impinges on hippocampal muscarinic and nicotinic ACh receptors. As described later in the chapter, ACh receptors modify neuronal activity, through multiple signalling cascades characterized by different spatial location and time course (Teles-Grilo Ruivo LM & Mellor JR, 2013).

Muscarinic ACh receptors

The muscarinic ACh receptors (mAChRs) are members of the class of heptahelical G protein–coupled receptors (GPCRs). Five main subtypes of muscarinic receptors (M1–M5) have been identified. Their localization in the hippocampal formation was investigated using subtype-specific antibodies (Levey, Edmunds, Hersch, Wiley, & Heilman, 1995). Each receptor subtype, differentially localized in the hippocampal areas, modulates a variety of processes, including long term synaptic plasticity (Origlia et al., 2006). M1 receptors are widely expressed on the somata and dendrites of the pyramidal neurons of CA1-CA3 areas and on granule cells of the dentate gyrus. Some M3 receptors are located on pyramidal neurons, on the neuropil of the stratum lacunosum molecularis and the outer third of the molecular layer of dentate gyrus; M2 and M4 subtypes are located presynaptically in several bands of fibers, and postsynaptically in non-pyramidal neurons and in the inner layer of the molecular layer. As a consequence of their pre- and postsynaptic location, mAChRs have different impacts on neuronal activity. Presynaptic mAChRs (M_2, M_4) are coupled to $G_{i/o}$ and inhibit voltage-gated Ca^{2+} channels, decrease cAMP-mediated signaling and inhibit neurotransmitter release at cholinergic (Vannucchi & Pepeu, 1995; Vannucchi, Scali, Kopf, Pepeu, & Casamenti, 1997; Zhang et al., 2002), GABAergic and glutamatergic terminals (Russo, Marchi, Andrioli, Cavazzani, & Raiteri, 1993; Gonzales, Pare, Wichmann, & Smith, 2013; Szabo, Holderith, Gulyas, Freund,& Hajos, 2010; Dasari & Gulledge, 2011). Postsynaptic mAChRs (M_1, M_3, M_5) are coupled to $G_{q/11}$ and potentiate NMDA currents (Markram & Segal, 1990c; Marino, Rouse, Levey, Potter, & Conn, 1998; Fernandez De Sevilla, Nunez, Borde, Malinow, & Buno, 2008), modulate voltage-dependent Ca^{2+} currents (Toselli, Lang, Costa, & Lux, 1989) and activate phopholipase C, inositol trisphosphate and

increase of intracellular Ca^{2+} concentration (Power & Sah, 2002; Gulledge & Kawaguchi, 2007). Furthermore, mAChRs coupled to $G_{q/11}$ inhibit K^+ conductances, causing membrane depolarization and increasing input resistance (Brown & Adams, 1980; Halliwell & Adams, 1982; Cole & Nicoll, 1984; Madison, Lancaster,& Nicoll, 1987; Buchanan, Petrovic, Chamberlain, Marrion, & Mellor, 2010; Giessel and Sabatini, 2010).

The literature on the disruptive effect of muscarinic antagonists, namely scopolamine and atropine, on cognitive processes is extensive and has been largely reviewed (Izquierdo, 1989; Klinkenberg & Blokland, 2010; Brown, 2010; Graef, Schoknecht, Sabri, & Hegerl, 2011). We shall only focus on some examples taken from the literature that corroborate the role of the basal forebrain cholinergic neurons innervating the hippocampus on IA memory formation (Table 1). In several IA studies (Wiener and Messer, 1973; Rush, 1988; Quirarte et al., 1994; Nomura, Nishiyama, Saito, & Matsuki, 1994, Eidi, Zarrindast, Eidi, Oryan & Parivar, 2003; Giovannini et al., 2005; Lana et al., 2013) it has been demonstrated that systemic, intracerebral or intrahippocampal administration of scopolamine before training is effective in impairing recall at 1 h or 24 h after training (Table 1). Nevertheless, the level of shock intensity interferes with the effect of scopolamine on passive avoidance retention, as shown by Quirarte et al. (1994). A dose of 8 mg/kg, IP caused amnesia using low foot shock conditions, but it was not effective when high level of foot shock was employed. Furthermore, intra-hippocampal administration of the muscarinic agonist oxotremorine or of the muscarinic toxin MT2, a highly selective agonist for M1 receptors from the venom of the snake *Dendroaspis angusticeps,* enhances retention of an inhibitory avoidance (Izquierdo et al. 1992; Jeruzalinsky, Cervenansky, Walz, Bianchin, & Izquierdo, 1993). These effects can be antagonized by scopolamine (Jeruzalinsky, Cervenansky, Walz, Bianchin, & Izquierdo, 1993), and led the authors to postulate that the m1 receptor of the dorsal hippocampus is directly involved memory formation of this task (Jerusalinsky et al., 1993).

Nicotinic ACh receptors

The nicotinic ACh receptors (nAChRs) are a family of ACh-gated ion channels formed by different subtypes, each with specific anatomical distribution as well as different pharmacology and physiology. Twelve neuronal subunits have been described including 9 α (α2-α10) and 3 β (β2-β4) subunits. Only the α subunits contain the binding site for ACh (Alkondon & Albuquerque, 1993). The combination of these subunits defines the function and affinity of the receptor for specific ligands (Sudweeks & Yakel, 2000). In the hippocampus, α7, α4β2 and α3β4 nAChRs have been detected (Teles-Grilo Ruivo LM &

Mellor JR, 2013). The α4β2 subtype is present on the somata of excitatory neurons and presynaptically on GABAergic terminals. The α3β4 subtype was found on glutamatergic and GABAergic terminals. The α7 receptors are located presynaptically and postsynaptically at both glutamatergic and GABAergic synapses and postsynaptically at cholinergic synapses (Ji & Dani, 2000; Alkondon & Albuquerque, 2004). The α7 nAChR, in addition to its ionotropic activity, is associated with metabotropic activity coupled to Ca^{2+}-regulated second messenger signalling (Berg & Conroy, 2002). Activation of nAChRs results in direct Ca^{2+} influx through the channel pore and rapid membrane depolarization. The precise Ca^{2+} permeability of receptors depends on the subunit composition with α7 being the most permeable. Ca^{2+} accumulation in presynaptic terminals facilitates neurotransmitter release (Lena, Changeux, & Mulle, 1993; McGehee, Heath, Gelber, Devay, & Role, 1995; Wonnacott, 1997; Fu, Liou, & Chen, 1998; Tang et al., 2011). Postsynaptically, cations flux through nAChRs mediates fast excitatory synaptic responses (Frazier et al., 1998; McQuiston & Madison, 1999; Ji & Dani, 2000; Alkondon & Albuquerque, 2001; Kawai, Zago & Berg, 2002; Wanaverbecq, Semyanov, Pavlov, Walker, & Kullmann, 2007; Bell, Shim, Chen, & McQuiston, 2011; Gu & Yakel, 2011; Tang et al., 2011). Fast membrane depolarization triggers activation of voltage-gated Ca^{2+} channels, increase of second messenger cAMP (Margiotta, Berg, & Dionne, 1987; Sargent, 1993) and Ca^{2+} release from intracellular stores (Vijayaraghavan, Pugh, Zhang, Rathouz, & Berg, 1992; Sharma & Vijayaraghavan, 2003). Ca^{2+} influx through nAChRs activates Ca^{2+}-dependent Cl^- conductances (Mulle, Choquet, Korn, & Changeux, 1992; Vernino, Amador, Luetje, Patrick, & Dani, 1992), which oppose the depolarization caused by nAChR opening. nAChRs differentially modulate neuronal excitability, depending on the target cell, and the strength and timing of the cholinergic input (Frazier et al., 1998; Ji & Dani, 2000; Alkondon & Albuquerque, 2004).

Intrahippocampal administration of the nAChR agonist nicotine facilitates working memory (Felix & Levin, 1997; Levin, McClernon, & Rezvani, 2006), while intrahippocampal administration of the nAChR antagonists dihydro-b-erythroidine, methyllycaconitine (Felix & Levin, 1997; Levin, Bradley, Addy, & Sigurani, 2002) or mecamylamine (Ohno, Yamamoto, & Watanabe, 1993) impairs working memory. Nicotinic receptors in the CA1 region of the hippocampus have been involved in both STM and LTM formation, and in retrieval processes of an IA response in rats, suggesting that nAChRs have a modulatory role in different types and phases of memory (Marti, Ramirez, Dos Reis, & Izquierdo, 2004). Systemic nicotine administration 15 min prior to a retrieval test ameliorates IA memory. This effect is opposed by the centrally acting antagonist mecamylamine but not by the peripherally acting antagonist

hexamethonium or the muscarinic antagonist atropine all given IP (Zarrindast, Sadegh, & Shafaghi, 1996). Post-training intracerebroventricular infusions of ACh or nicotine have been shown to enhance inhibitory avoidance. This effect is reduced by coinfusion of scopolamine (Eidi, Zarrindast, Eidi, Oryan, & Parivar, 2003).

Using microdialysis in freely moving rats, it was shown that hippocampal memory processes are associated with a marked increase in ACh release (Ragozzino, Pal, Unick, Stefani, & Gold, 1998; Stancampiano, Cocco, Cugusi, Sarais, & Fadda, 1999; Giovannini et al., 2001b; Giovannini et al., 2005; Bianchi et al., 2003). Behavioural conditions that induce arousal, require attention and lead to information acquisition and memory formation, are associated and supported by activation of the forebrain cholinergic system (Demeter & Sarter, 2013).

Disruption of the hippocampal cholinergic input in animals further demonstrates the importance of this structure in cognitive processes. Inhibition of ACh synthesis induced by hemicholinium-3 ICV administration, a selective inhibitor of high-affinity choline uptake (Gardiner, 1961) leads to memory consolidation impairment in the IA task in mice (Boccia, Acosta, Blake, & Baratti, 2004). Power &McGaugh (2002), using the nonselective cholinergic excitotoxin phthalic acid injected in the NBM, found that phthalic acid-lesioned animals showed a significant reduction of inhibitory avoidance learning. This impairment could be rescued by ipsilateral infusions of the muscarinic agonist oxotremorine or the acetylcholinesterase inhibitor physostigmine. Furthermore, intra-hippocampal injection of the muscarinic receptor antagonist scopolamine impairs memory acquisition in a IA task (Giovannini et al., 2005) and spatial discrimination learning in the Morris water maze (Blokland, Honig, & Raaijmakers, 1992). IP administration of atropine, a central muscarinic antagonist, completely prevents the facilitatory effects of the central β2-adrenoreceptor agonist, clenbuterol also given IP (Introini-Collison & Baratti, 1992), suggesting an interaction between central adrenergic and cholinergic mechanisms in the IA response in mice. Furthermore, selective lesion of the medial septal and diagonal band cholinergic neurons resulted in deficits in spatial strategies used to locate a spatial goal in the Morris water maze (Janis, Glasier, Fulop, & Stein, 1998). Lesions of the cholinergic and/or GABAergic neurons in the medial septum and diagonal band showed that GABAergic and cholinergic septohippocampal neurons both contribute to memory stabilization (Lecourtier et al., 2011) whereby GABAergic processes could be engaged at an earlier stage than cholinergic ones during system consolidation of a spatial memory. Disruption of the GABAergic neurons of the medial septum and diagonal band impairs ACh efflux and

working memory under the heavy memory load of a delayed non matching to position task, but does not alter hippocampal ACh efflux and easier memory tasks (Roland et al., 2014).

The toxin 192 IgG-saporin at present is the most convenient tool to induce selective cholinergic denervation (Waite & Thal, 1996; Wiley, Oeltmann & Lappi 1991). 192 IgG-saporin is constituted of the monoclonal antibody 192 IgG which has a low affinity to nerve growth factor (NGF) receptor p75 present on cholinergic neurons and saporin, a ribosome inactivating toxin. The 192 IgG-saporin binds to the p75 NGF receptors, is internalized and retrogradely transported to the soma, where it is cleaved. Saporin disrupts the ribosomal function, thus leading to cell death (Wiley, Oeltmann & Lappi 1991).

The intracerebral injection of this toxin to disrupt cholinergic neurons has given controversial results. Although 192 IgG-saporin brings about selective and significant cholinergic damage of the NBM, only modest deficits in mnemonic tasks have been reported (Torres et al., 1994; Baxter et al., 1996). For instance, 192 IgG-saporin lesions had no effect on inhibitory avoidance learning (Power, Thal & McGaugh, 2002). Some of the authors explained the unexpectedly modest effect of immunotoxin lesions on memory paradigms with the possible existence of compensatory mechanisms after the lesions (Lacroix, White & Feldon, 2002; de Bruin, Ellenbroek, van Luijtelaar, Cools & Stevens, 2001), or with the immunotoxin selective effect on cortical projections and comparative lack of effect on amygdalopetal cholinergic projections (Power, Thal & McGaugh, 2002). More recently it has been shown that only ICV lesions, but not NBM lesions using 192 IgG-saporin lead to memory impairments in passive avoidance and Morris water maze tasks (Garcia-Alloza et al, 2006). Also, rats with immunotoxic lesions of cholinergic neurons in the MS/VDB, are unimpaired in a test of ‘‘episodic-like’’ memory (Easton, Fitchett, Eacott & Baxter, 2011). On the other hand, very recently it has been demonstrated that 192 IgG-saporin impairs spatial learning (Rastogi, Unni, Sharma, Rao Laxmi & Kutty, 2014). Others demonstrated that 192 IgG-saporin causes basal forebrain cholinergic depletion and impairs working memory, spatial discrimination, social novelty preference (Cutuli et al., 2013), and these effects are prevented by administration of donepezil, an indirect cholinomimetic drug. The different types of behavioural tests used and memories studied as well as the participation of other neutroansmitter systems in learning and memory mechanisms may explain the contrasting effects of 192 IgG-saporin lesions described in the literature.

3. The cholinergic system and ERK transduction pathway in memory formation

ACh, released in the proximity of depolarized neurons, brings about a long-lasting potentiation of the postsynaptic membrane (Tremblay, Warren, & Dykes, 1990; Metherate, 1998). Stimulation of muscarinic/nicotinic receptors subtypes present on neurons (Rosenblum, Futter, Jones, Hulme, & Bliss, 2000; Dineley et al., 2001; Berkeley et al., 2001; Giovannini et al., 2008) may create conditions favourable for neuronal plasticity, initiating a network of signals that activate several intracellular transduction pathways including the ERK pathway (Gutkind, 1998). ERK is also activated by glutamate through metabotropic (Peavy & Conn, 1998) or ionotropic glutamate receptors (Zhu, Qin, Zhao, Van Aelst, & Malinow, 2002; Krapivinsky et al., 2003), by noradrenaline through β-adrenergic receptors (Williams, Zhong, & Minneman, 1998; Winder et al., 1999; Watabe, Zaki, & O'Dell, 2000), by other neurotransmitters (Drutel et al., 2001; Giovannini et al., 2003), and growth factors (Castillo & Escobar, 2011) and by the sex steroid hormones 17beta-estradiol and progesterone (Harburger, Saadi, & Frick, 2009; Orr, Rubin, Fan, Kent, & Frick, 2012).

It has been demonstrated that combined stimulation of mAChR and β-adrenergic receptors synergistically activate ERK which can act as a coincidence detector (to decode the simultaneous engagement of different receptors) and as a signal integrator (that encodes this information in a spatially and temporally distinct biological signals) (Watabe, Zaki, & O'Dell, 2000; Sweatt, 2001; Geetha et al., 2011), thus activating a cascade of intracellular processes that lead to synaptic plasticity and learning. Indeed, ERKs are placed at a strategic position allowing crosstalk between different arrays of signals and signal transduction pathways.

ERK is localized in the soma and dendritic trees of neurons in the neocortex, hippocampus, striatum, and cerebellum (Fiore et al., 1993). Phosphorylation of ERK by its upstream kinase MEK is necessary for the formation of different types of learning and memory (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Blum, Moore, Adams, & Dash, 1999; Kaminska, Kaczmarek, Zangenehpour, & Chaudhuri, 1999; Walz et al., 2000; Cammarota et al., 2000). The first direct evidence that ERK is involved in memory processes *in vivo* was reported in a seminal paper published by Sweatts' group (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998). These findings (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998) were confirmed and expanded using inhibitors of ERK activation in the rat (PD098059 or U0126 injected intracerebrally) (Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999; Schafe et al., 2000). Later studies showed that activation of the ERK hippocampal pathway is required for long-term fear memory (Giovannini et al., 2003; Apergis-Schoute, Debiec, Doyere, LeDoux, & Schafe, 2005).

Downstream effectors of ERK activation

Within minutes from activation, a fraction of phospho-ERK translocates to the nucleus (Davis, Vanhoutte, Pages, Caboche, & Laroche, 2000), where it can modify gene expression by transcriptional control (Xia, Dudek, Miranti, & Greenberg, 1996; Impey et al., 1998). It is likely that ERK participates in different forms of neuronal plasticity by virtue of its ability to regulate both transcription at the nuclear level (for a review, see Impey et al., 1998; Chang & Karin, 2001) and translation in the dendrites (Chen, Rojas-Soto, Oguni, & Kennedy, 1998; Kim, Liao, Lau, & Huganir, 1998; Flood et al., 1998). The former effect is consistent with the participation of ERK in memory formation through protein synthesis (Chwang, Arthur, Schumacher, & Sweatt, 2007) whereas the translational effects occur through phosphorylation and changes in local synaptic mechanisms (English & Sweatt, 1996; English & Sweatt, 1997; Impey et al., 1998; Giovannini et al., 2001a). Thus, it seems plausible that ERK participates in both forms of memory, by modifying the existing proteins that determine synaptic behavior, and/or by regulating the expression of proteins necessary for the long-term maintenance of synaptic changes. Some of these latter effects are thought to reflect ERK-dependent activation of transcription factors such as CREB and Elk-1 (Treisman, 1995; Treisman, 1996; for review see Sweatt, 2001).

After activation, the fraction of ERK that remains in the dendrites is extensively phosphorylated (Impey et al., 1998; Winder et al., 1999; Giovannini et al., 2005) pointing to an involvement of ERK in the activation of downstream cytoplasmic proteins such as mTOR (Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007), ribosomal S6 kinase2, RSK2 (Poteet-Smith, Smith, Lannigan, Freed, & Sturgill, 1999), that regulate translational efficiency (Grewal, York, & Stork, 1999). Other extranuclear substrates for ERK include components of the postsynaptic signalling network such as phospholipase A_2 (Xu et al., 2002), SynGAP (Muthalif, Benter, Uddin, & Malik, 1996; Chen, Rojas-Soto, Oguni, & Kennedy, 1998; Kim, Liao, Lau, & Huganir, 1998), and several microtubule-associated proteins (MAP), such as MAP-1, MAP-2, MAP-4, and Tau (Seger & Krebs, 1995). Furthermore, the postsynaptic density, a subsynaptic complex in which much of the postsynaptic signalling occurs , includes ERK2, MEK, and the phosphatase (MKP2) (Husi, Ward, Choudhary, Blackstock, & Grant, 2000). Furthermore, dendritic phospho-ERK appears to play an important role in regulating K^+ channels, particularly in the phosphorylation of the pore-forming α subunit of Kv4.2 channels. Likely, this role contributes to dendritic information processing and increasing membrane excitability (Yuan, Adams, Swank, Sweatt, & Johnston, 2002; Watanabe, Hoffman, Migliore, & Johnston, 2002; Morozov et al., 2003; Sweatt, 2004).

Adding an even higher level of complexity to the involvement of ERK in memory mechanisms, it has been shown that the ERK cascade is involved in epigenetic mechanisms (Berger, Kouzarides, Shiekhattar, & Shilatifard, 2009) in the hippocampus, such as downstream histone H3 acetylation and phosphorylation *via* nuclear kinases (Levenson et al., 2004; Chwang, O'Riordan, Levenson, & Sweatt, 2006; Chwang, Arthur, Schumacher, & Sweatt, 2007). Stimulation of ERK signalling (Levenson et al., 2004) produces gene- and histone-specific changes in post translational modifications, indicating that distinct signalling cascades may establish precise histone codes that correspond to particular types of memory (Graff, Kim, Dobbin, & Tsai, 2011). Together, these findings support the possibility that ERK may play a role in memory both through nuclear and local synaptic mechanisms dependently and/or independently on gene transcription.

Role of ERK1 and ERK2

Two isoforms of ERK are present in cells, ERK1 (p44MAPK) and ERK2 (p42MAPK), which have similar distribution in the brain, although the amount of ERK1 in neurons of rat hippocampus appears to be considerably lower than that of ERK2 (Kanterewicz et al., 2000; Giovannini et al., 2001a). The two isoforms share about 90% homology (Boulton et al., 1990) and have the same substrate specificity *in vitro*, but their role *in vivo* remains to be elucidated. It is still not fully understood whether both isoforms are equally involved in learning and memory mechanisms. Several groups have found that in neurons ERK1 and ERK2 are selectively regulated by different stimuli (Bading & Greenberg, 1991; Fiore, Murphy, Sanghera, Pelech, & Baraban, 1993; English & Sweatt, 1996; Giovannini et al., 2001a), and it has been suggested that only ERK2 plays a key role in synaptic plasticity and memory consolidation (Sweatt, 2001). Knockout (KO) mice for ERK1 and ERK2 have been generated and, whereas ERK1 KO mice are viable and appear to be neurologically normal (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001), ERK2 KO mice are embryonic lethal at day 6.5 (Yao et al., 2003; Saba-El-Leil et al., 2003). Therefore the two isoforms must have some important different function, at least early in mouse embryonic development (Saba-El-Leil et al., 2003). Selcher and coworkers demonstrated in KO mice that ERK1 is not required for emotional learning whereas ERK2 has a predominant role in synaptic plasticity underlying learning and memory processes (Selcher, Nekrasova, Paylor, Landreth, & Sweatt, 2001). It has also been shown (Mazzucchelli et al., 2002) that in ERK1 KO mice, STM is retained but there is a marked enhancement of LTM in a one-trial IA task. The view that the ERK2 isoform exerts a pivotal role in LTM modulation is supported also by the results of a

reconsolidation study (Cestari, Costanzi, Castellano, & Rossi-Arnaud, 2006) in which administration of SL327, an inhibitor of ERK activation, impaired memory reconsolidation not only in wt mice, but also in ERK1 KO mice. Altogether, these results clearly show a central role for ERK2 activation in memory reconsolidation processes in mice (Cestari, Costanzi, Castellano, & Rossi-Arnaud, 2006). It has also been suggested that ERK1 has a physiological inhibitory role on MEK (Mazzucchelli et al., 2002), thus limiting ERK1/2 activation. Some possible explanations for the selective activation of ERK2 in learning and memory mechanisms are its specific activation by upstream kinases, compartmentalization, differences in the brain structures involved, and binding to scaffolding proteins through highly specific docking sites (Sharrocks, Yang, & Galanis, 2000; Enslen & Davis, 2001), but so far there is no compelling evidence for any of these.

ERK activation in IA memory

As already mentioned, activation of the basal forebrain cholinergic pathway during memory acquisition, and the subsequent release of ACh, leads to stimulation of mAChRs that in turn trigger ERK activation either via PKC (Yasoshima & Yamamoto, 1997) or PYK2 (Lev et al., 1995). More recently, it has been demonstrated that nicotine may enhance hippocampus-dependent learning, most likely by impinging on α4β2 nAChRs and activating intracellular PKA and ERK pathways. Indeed, administration of the PKA inhibitor PKI 14-22 amide in the dorsal hippocampus (Gould et al., 2014) or an ERK inhibitor (SL327, administered IP) (Raybuck & Gould, 2007), at doses too low to impair learning *per se*, blocks learning facilitation elicited by nicotine. This suggests that nicotine administration during learning increases PKA and ERK activation and, if this increase is blocked, learning is impaired (Gould & Leach, 2014). Nicotine administered IP prior to learning increases CREB phosphorylation at the Jnk1 promoter region (Kenney, Poole, Adoff, Logue, & Gould, 2012) and Jnk1 expression in the hippocampus (Kenney, Florian, Portugal, Abel, & Gould, 2010). Thus, the ability of nicotine to modify cell signalling cascades involved in learning and to express additional signalling cascades may explain why nicotine administration is associated with the formation of a strong drug-context memory contributing to the drug seeking behaviour (Walters, Cleck, Kuo, & Blendy, 2005; Wilkinson & Bevins, 2008; Portugal & Gould, 2009; Gould & Leach, 2014).

Few investigations exist which directly correlate the increase in ACh release to performance of an IA task in the rat. We demonstrated (Giovannini et al., 2005) that IA acquisition initiates a cascade of events that activates hippocampally-projecting cholinergic

neurons. This is revealed by an increase in ACh release during and immediately after acquisition (Giovannini et al., 2005), by the activation of ERK in CA1 hippocampal neurons and memory formation. We showed (Giovannini et al., 2005) that administration of the mAChRs antagonist scopolamine (IP, or locally into the hippocampus) prior to training, and of ERK inhibitors U0126 and PD98059 (locally into the hippocampus), cause both inhibition of ERK activation and amnesia, demonstrating that both ACh release and ERK activation are necessary for IA STM formation. These results indicate that an increase in ACh release acting on postsynaptic mAChRs triggers an intracellular signalling cascade that activates ERK and further downstream effectors leading to memory encoding.

A time-window for ERK activation to be efficacious must exist, since it has been demonstrated that, at least in spatial memory, delayed infusion of MEK inhibitors U0126 and PD98059 does not interfere with long term spatial memory retention (Blum, Moore, Adams, & Dash, 1999). Since the neuronal ERK cascade can function as a biochemical coincidence detector, being activated simultaneously by β-adrenergic receptors and mAChRs in the hippocampus (Watabe, Zaki, & O'Dell, 2000), it is feasible that neurotransmitter systems other than the cholinergic may be concomitantly activated during acquisition. It is also possible that blocking one of the converging pathways is sufficient to inhibit the entire ERK cascade and the encoding of the IA memory. These findings show that an aversive experience such as the exposure to a single footshock initiates a cascade of events that, through the activation of hippocampally-projecting cholinergic neurons, promotes the activation of the ERK pathway and leads to the formation of STM of that event.

Izquierdo's group (Cammarota et al., 2000) demonstrated that learning of IA is associated with a similar, NMDA dependent, activation of both ERK1 and ERK2 in the rat hippocampus. In a further series of papers (Walz et al., 1999; Walz et al., 2000) the authors found that inhibition of ERK activation by PD 098059 in the CA1 region, entorhinal cortex, parietal cortex or amygdala impaired retention of the IA when tested up to 6 h after training, with a differential time course in the different brain regions. The authors thus concluded that the ERK pathway is involved in the IA post-training memory consolidation, with a different time-course in the hippocampus, amygdala, entorhinal cortex or parietal cortex of rats (Walz et al., 2000). It seems that activation of ERK correlates mostly with the aversive, emotional, component of IA acquisition since it was previously found that ERK is activated by mild electric footshocks similar in intensity and length to that employed as an aversive stimulus during IA training (Bevilaqua et al., 2006), but not by exposure to the training box in the absence of the footshock (Alonso, Viola, Izquierdo, & Medina, 2002). More recently,

experiments from the same group (Igaz, Bekinschtein, Izquierdo, & Medina, 2004) demonstrated that single trial step-down IA causes an increase of total ERK mRNA 3 h, and ERK2 protein upregulation 24 h after training. These results are interesting in that they reveal that ERK activity can possibly be modulated in different behavioural tasks not only by activation, but also by increased protein expression.

ERK activation in STM and LTM formation

Although some authors showed that ERK activation participates in LTM but not STM (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Berman, Hazvi, Rosenblum, Seger, & Dudai, 1998; Blum, Moore, Adams, & Dash, 1999), data from our and other laboratories indicate that a rapid and transient activation of ERK participates in the molecular mechanisms underlying IA STM formation (Giovannini et al., 2005; Walz et al., 1999; Izquierdo et al., 2000; Alonso, Viola, Izquierdo, & Medina, 2002). The effects of inhibitors of ERK activation injected immediately post training into CA1 or entorhinal cortex on STM and LTM show an interesting mirror image. In CA1, the role of ERK appears to be restricted mainly to STM formation. Simultaneously, in the entorhinal cortex the activation of this pathway impairs STM formation but is necessary for LTM formation. Studies with the MEK inhibitor PD98059, injected intracortically at different times after training, point to a role of ERK in LTM consolidation (Walz et al., 1999; Walz et al., 2000). These data suggest that the ERK pathway plays a complex regulatory role in synaptic plasticity. It is linked at different levels with the PKC, CaMKII, and PKA cascades (Bhalla & Iyengar, 1999; Lisman & Fallon, 1999) which are all crucial for LTM and, depending on brain structure and post training time, also for STM (Cammarota, Bevilaqua, Medina, & Izquierdo, 2007).

In agreement with the literature (Walz et al., 1999; Walz et al., 2000; Alonso, Viola, Izquierdo, & Medina, 2002), the duration of ERK activation is limited to a restricted time window after training, indicating that acquisition of aversive experiences is associated with a rapid and short lasting activation of ERK. According to these data, it seems that an inherent signal termination process limits the duration of ERK activation (Pouyssegur, Volmat, & Lenormand, 2002), which depends upon a balance between the activity of kinases and phosphatases (Kwak, Hakes, Martell, & Dixon, 1994; Misra-Press, Rim, Yao, Roberson, & Stork, 1995; Muda et al., 1996; Boschert, Dickinson, Muda, Camps, & Arkinstall, 1998). Many of these latter proteins are induced by stimuli that also activate ERK and participate in a negative feedback control of ERK activity (Paul, Nairn, Wang, & Lombroso, 2003) in which ERK itself determines the duration of its own activation by in turn activating phosphatases

(Pouyssegur, Volmat, & Lenormand, 2002). Since ERK can function as a biochemical signal integrator in response to extracellular signals in neurons (Watabe, Zaki, & O'Dell, 2000), the ramifications of ERK-dependent signalling are determined, in part, by the duration of ERK phosphorylation itself and it may not be surprising to find that the duration of its activation is tightly regulated. Indeed, short-term activation of ERK triggers cell differentiation in PC12 cells, while prolonged activation results in cell proliferation (Traverse, Gomez, Paterson, Marshall, & Cohen, 1992). Repeated depolarizations, rather than a single depolarization, cause sustained ERK activation, which is essential for new spine formation in neuron culture, since MEK inhibition with PD98059 prevents both ERK activation and spine formation events (Wu, Deisseroth, & Tsien, 2001).

Baseline ERK activation decreases in basal conditions in the hippocampus of TgCRND8 mice, an early-onset transgenic mouse model of Alzheimer's Disease (AD) (Chishti et al., 2001;Bellucci et al., 2006). Cholinergic stimulation *ex vivo* strongly increases ERK activation in the cell bodies of CA1 pyramidal neurons and of DG granule cells of wild type (wt) mice, showing that activation of ERK in these neurons is downstream of cholinergic activation (Bellucci et al., 2006). This effect is significantly smaller in the hippocampus of transgenic mice, indicating a possible mechanism responsible for the memory deficits present in TgCRND8 mice (Bellucci et al., 2006). Most interestingly, the cholinergic agonist carbachol induced a much lower activation of ERK in TgCRND8 mice hippocampal slices *in vitro* than in slices from wt littermates (Giovannini et al., 2008). This effect may be ascribed to modifications upstream of ERK, such as a decrease in the number of mAChRs which are significantly reduced in TgCRND8 mice (Bellucci et al., 2006). Thus, these findings offer a molecular basis for memory disruption in AD, since memory requires proper functioning of the basal forebrain cholinergic neurons (Sarter & Bruno, 1997b), and ERK2 activation (Atkins, Selcher, Petraitis, Trzaskos, & Sweatt, 1998; Blum, Moore, Adams, & Dash, 1999; Kaminska, Kaczmarek, Zangenehpour, & Chaudhuri, 1999; Walz et al., 2000; Cammarota et al., 2000; Adams & Sweatt, 2002).

As mentioned above, it appears (Pouyssegur, Volmat, & Lenormand, 2002) that the kinetics and duration of ERK activation may play an important role in influencing its effect on cell fate. Obviously, differential durations of ERK activation are regulated by different molecular players (York et al., 1998; Corbit, Foster, & Rosner, 1999) and can elicit unique gene expression profiles, and/or protein translation which consequently result in different cellular functions (Marshall, 1995) and final cellular outcomes. The possibility that sustained ERK can activate death programs independent of caspases in neurons (Subramaniam et al.,

2004) suggests that ERK activation is involved in non-apoptotic modes of neuronal death. On the contrary, activation of ERK and Akt has been found to confer neuroprotection in several models of apoptosis (Hetman, Kanning, Cavanaugh, & Xia, 1999). Similarly, both Akt and ERK have been reported to play a role in regulating hippocampal neurogenesis (Aberg et al., 2003).

4. The Cholinergic system and mTOR pathway in memory formation

LTM formation require protein synthesis, and direct translation in the synapto-dendritic compartment represents a mechanism that can produce rapid changes in protein content in response to synaptic activity (Bailey, Kandel, & Si, 2004; Kelleher, III, Govindarajan, Jung, Kang, & Tonegawa, 2004; Hoeffer & Klann, 2010).

The Mammalian Target of Rapamycin (mTOR), is an evolutionary conserved high molecular weight serine-threonine protein kinase that regulates cell growth, proliferation and survival (Martin & Hall, 2005) by increasing protein translation. In the brain, mTOR regulates protein translation in response to neuronal activity, thereby modulating synaptic plasticity and LTM formation (Kelleher, III, Govindarajan, Jung, Kang, & Tonegawa, 2004). Downstream targets of mTOR include p70S6K, and eukaryotic Elongation Factor 1A and 2 (eEF1A and eEF2), which are mostly involved in ribosome recruitment to mRNA, the eukaryotic initiation factor 4E binding proteins (4E-BPs), which regulate both the initiation and elongation phases of translation (Hay & Sonenberg, 2004).

Activation of mTOR by growth factors is well documented (Hay & Sonenberg, 2004; Slipczuk et al., 2009). It has also been demonstrated that mTOR can be activated by GPCRs (Wang & Proud, 2002; Arvisais, Romanelli, Hou, & Davis, 2006) and in particular it was demonstrated in a neuroblastoma cell line *in vitro* that the mTOR pathway is activated by mAChRs (Slack & Blusztajn, 2008). Furthermore, mTOR activation is downstream of nAChRs in cultured non–small-cell lung carcinoma cells (Zheng, Ritzenthaler, Roman, & Han, 2007). The above results indicate that mTOR can be downstream of cholinergic receptors.

The mTOR pathway was first implicated in synaptic plasticity when it was shown that rapamycin, a selective inhibitor of mTOR Complex1 (mTORC1) activity (Casadio et al., 1999; Takei, Kawamura, Hara, Yonezawa, & Nawa, 2001; Hay & Sonenberg, 2004; Takei et al., 2004) blocks long-term facilitation in *Aplysia* (Casadio et al., 1999) In addition, mTOR-dependent activation of dendritic ribosomal protein kinase (p70S6K) was shown to be

necessary for the induction phase of protein-synthesis-dependent synaptic plasticity (Cammalleri et al., 2003).

Whereas several studies have examined the effects of mTOR inhibition on synaptic plasticity *in vitro* (Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007), few have examined the role of mTOR in learning and memory *in vivo* (Parsons, Gafford, & Helmstetter, 2006; Bekinschtein et al., 2007). The latter authors were the first to demonstrate that acquisition or consolidation of fear memories in the hippocampus or amygdala require mTOR activity (Parsons, Gafford, & Helmstetter, 2006; Bekinschtein et al., 2007). Furthermore, central administration of rapamycin *in vivo* disrupts the formation of different types of memories (Tischmeyer et al., 2003; Parsons, Gafford, & Helmstetter, 2006; Dash, Orsi, & Moore, 2006; Schicknick et al., 2008; Sui, Wang, & Li, 2008; Belelovsky, Kaphzan, Elkobi, & Rosenblum, 2009).

Interestingly, it was reported that in cultured neurons and hippocampal slices from AD transgenic mice and in hippocampal slices from wt mice, exposed to exogenous Aβ1-42, the mTOR signalling pathway is inhibited (Ma et al., 2010). The mTOR dysregulation correlates with impairment in synaptic plasticity. On the contrary, upregulation of mTOR signalling by both pharmacological and genetic methods prevents Aβ-induced synaptic impairment, indicating that dysregulation of the mTOR pathway could play a role in the synaptic dysfunction that characterizes AD (Ma et al., 2010). As mentioned above, the effects of ACh on learning and memory in the hippocampus appear to be mediated mainly by mAChRs (Izquierdo et al., 1998b; Barros, Pereira, Medina, & Izquierdo, 2002; Giovannini et al., 2005), although there is evidence indicating that nAChRs have an important modulatory role (Decker, Brioni, Bannon, & Arneric, 1995; Marti, Ramirez, Dos Reis, & Izquierdo, 2004; Mitsushima, Sano, & Takahashi, 2013). Some years ago, it was demonstrated (Feig & Lipton, 1993) that activation of mAChRs stimulates new protein synthesis in hippocampal CA1 dendrites. Since then little progress has been made in understanding the role of local protein synthesis in mAChR-dependent synaptic plasticity. Recent results that demonstrated that the mTOR pathway is downstream of mAChRs and nAChRs (Zheng, Ritzenthaler, Roman, & Han, 2007;Slack & Blusztajn, 2008), link the cholinergic system to mTOR activation and to local protein synthesis via multiple MAPK- and/or PKC-dependent mechanisms. Specifically, M2 receptors utilize a MAPK-dependent mechanism to activate this pathway, whereas M3 receptors utilize either MAPK dependent or independent mechanisms, depending on cellular context (Slack & Blusztajn, 2008). Furthermore, in a

recent paper the authors demonstrate a fine regulation of mTOR and p70S6K by the muscarinic M4 receptor in PC12 cells (Chan, Wu, & Wong, 2009).

In a recent study from our laboratory, rapamycin (injected ICV) was used as a pharmacological tool to dissect the intracellular translational machinery activated by upstream signals in the acquisition and retrieval of an IA memory (Lana et al., 2013). We showed that mTOR and its downstream effector p70S6K are massively activated in most of CA1 pyramidal neurons at early times after acquisition of an IA memory (Lana et al., 2013). A fairly rapid and transient inactivation of mTORC1 and, consequently, of p70S6K by rapamycin impairs formation of LTM with no effect on STM, demonstrating that mTORC1 activation is necessary for LTM. These data are consistent with those reported by Hoeffer who demonstrated that rapamycin disrupts fear associated LTM formation 24 h, but not 3 h, after acquisition (Hoeffer et al., 2008).

An intriguing result (Lana et al., 2013) is the observation that 1 h after administration of the mAChR antagonist scopolamine, activation of mTOR and p70S6K is increased. Presynaptic M2/M4 mAChRs, located on septo-hippocampal cholinergic terminals (Quirion et al., 1995), act as inhibitory autoreceptors (Raiteri, Leardi, & Marchi, 1984; Douglas, Baghdoyan, & Lydic, 2002; Zhang et al., 2002) and their blockade by scopolamine (Figure 1) massively increases ACh release (Scali, Vannucchi, Pepeu, & Casamenti, 1995), which, in the presence of the non-selective muscarinic antagonist scopolamine, only binds to postsynaptic nAChRs leading to activation of the mTOR pathway. The link of nAChR to the mTOR pathway has been demonstrated in other systems (Zheng, Ritzenthaler, Roman, & Han, 2007; Sun et al., 2009) and, as reported above, the involvement of nAChR to mediate ACh postsynaptic responses in the hippocampus is substantiated by several studies (Marti, Ramirez, Dos Reis, & Izquierdo, 2004; Bell, Shim, Chen, & McQuiston, 2011; Gu & Yakel, 2011). An alternative explanation of the increase in mTOR activation following scopolamine may be that the large and long-lasting increase of ACh release evoked by scopolamine (Scali, Vannucchi, Pepeu, & Casamenti, 1995) may in time overcome the postsynaptic antagonistic effect of the muscarinic competitive antagonist, with the ensuing activation of intracellular pathways and consequent increase of the mTOR intracellular signalling. This may trigger protein translation, presumably responsible for LTM formation (Bekinschtein et al., 2010). In a recent study (Mitsushima, Sano, & Takahashi, 2013), it was found that ACh mediates learning-induced strengthening at excitatory and inhibitory synapses through distinct sets of AChRs. Activation of mAChRs mediates the IA learning through the incorporation of AMPA-type glutamate receptors into hippocampal CA3-CA1 synapses. IA learning also

strengthens inhibitory hippocampal synapses through the activation of nAChRs but not mAChRs. These data reveal novel molecular and cellular mechanisms of learning-dependent synaptic plasticity. Thus, ACh balances the excitatory and inhibitory synaptic inputs onto CA1 pyramidal neurons in IA learning through the activation of distinct sets of AChRs (Mitsushima, Sano, & Takahashi, 2013). Therefore, ACh function on any given circuit and intracellular pathways may depend on the specific expression of postsynaptic mAChRs versus nAChRs and upon the temporal dynamics of ACh levels in the synaptic cleft.

In Figure 1 the contribution of the cholinergic system and the downstream effectors ERK and mTOR in the formation of inibitory avoidance (IA) short term and long term memories (STM, LTM) is shown. ACh released by presynaptic terminal activates both muscarinic and nicotinic postsynaptic receptors (mAChRs, nAChRs). Postsynaptic mAChRs and nAChRs indirectly activate the intracellular pathways of ERK and mTOR, responsible, with different contribution, and different downstream effectors, for IA STM and LTM formation. Inhibitory mAChRs presynaptic receptors, blocked by muscarinic antagonists scopolamine and atropine, lead to massive increase of ACh release.

As shown in Figure 1, administration of muscarinic plus nicotinic antagonists *in vivo* blocks the scopolamine-induced increase of mTOR activation 1 h after administration (Lana et al., 2013). However, although mTOR is activated only at a later time (4 h) after administration of the drugs, LTM is still maintained (Lana et al., 2013). It is therefore possible that activation of mTOR at later times is sufficient to activate downstream effectors leading to LTM formation. The apparent discrepancy between the effect of muscarinic plus nicotinic antagonists on LTM formation and the decreased activation of mTOR and p70S6K may also be explained considering that several other neurotransmitter systems (Izquierdo et al., 1998c; Slipczuk et al., 2009) and other intracellular signalling pathways are involved in IA LTM formation (Khakpai, Nasehi, Haeri-Rohani, Eidi, & Zarrindast, 2012). A further explanation for this apparent discrepancy may come from data that demonstrate that in some instances mTORC1 activity regulates only a small component of total protein synthesis (Yanow, Manseau, Hislop, Castellucci, & Sossin, 1998; Choo, Yoon, Kim, Roux, & Blenis, 2008) and additional mTORC1-independent regulatory signals are required to induce memory since stimulation of mTORC1 probably generates a set of proteins important, but not sufficient for neuronal plasticity or memory (Graber, McCamphill, & Sossin, 2013).

A further demonstration that mTOR is downstream of mAChR activation derives from *in vitro* experiments on rat hippocampal slices. Carbachol significantly increases mTOR and p70S6K activation in CA1 pyramidal neurons *in vitro*. This effect is antagonized by the

mAChRs antagonist scopolamine (IP) and the nAChRs antagonist mecamylamine (ICV) administered together before carbachol (Lana et al., 2013).

Although at variance from data reported by some investigators (Marti, Ramirez, Dos Reis, & Izquierdo, 2004), these results (Lana et al., 2013) support the idea that scopolamine predominantly affects STM processes (Givens & Olton, 1995;Stanhope, McLenachan, & Dourish, 1995; Savage, Faust, Lambert, & Moerschbaecher, 1996;Estape & Steckler, 2002). These data are in accordance with data demonstrating that blockade of mAChRs by scopolamine given IP to animals is followed by an impairment of working memory and the disruption of recently acquired tasks, resembling the impairment of recent memory in humans, with no effect on spatial reference memory (Bartolini, Risaliti, & Pepeu, 1992) or maze performance in overtrained animals (Pazzagli & Pepeu, 1964).

A further refinement of the effect of mTOR activation on protein translation may come from the effects of downstream effectors of mTORC1, such as 4E-BP1, on protein translation. These effects are not limited simply to switching ‘off ’ or ‘on’ protein synthesis; they can also alter the range and the type of nascent proteins by mediating a switch between cap-dependent and cap-independent translation (Bove, Martinez-Vicente, & Vila, 2011).

Finally, recent reports indicate that LTM deficits can be associated with hyperactivation of the mTOR signalling pathway and an imbalance in protein synthesis (Bolduc, Bell, Cox, Broadie, & Tully, 2008). Puighermanal et al (2009) demonstrated that activation of the Cannabinoid receptor type 1 in the mouse hippocampus *in vivo* modulates the mTOR pathway, activating p70S6K and increasing protein translation. Contrary to what may be expected, in this case, an increase in protein translation seems to be responsible for the memory impairments caused by cannabinoid consumption (Puighermanal et al., 2009). In the same direction lead the findings showing that, although basal mTOR activity seems to be necessary for memory consolidation, an increase in mTOR signalling can disrupt memory processing. In patients with tuberous sclerosis and in animal models of this genetic disease, mutations that cause a reduction in Tuberous Sclerosis Complex1–Tuberous Sclerosis Complex2 activation and an increase in mTORC1 activity are associated with memory deficits (Ehninger et al., 2008).

Interplay between ACh and the ERK and mTOR pathways in IA memory encoding

A mechanistic model that may help explaining the integrated role of cholinergic activation and the downstream effectors ERK and mTOR in the formation of hippocampal inhibitory avoidance short term and long term memories is shown in Figure 1. The

cholinergic septo-hippocampal pathway is activated during acquisition of an IA memory (Giovannini et al., 2005) and ACh, released by presynaptic terminals, impinges on and activates both muscarinic and nicotinic postsynaptic receptors. Postsynaptic mAChRs indirectly activate the intracellular pathways of ERK and mTOR (dotted arrows), responsible, with different contributions and different downstream effectors, for IA STM and LTM formation (Giovannini et al., 2005; Lana et al., 2013). Inhibitory muscarinic presynaptic receptors, blocked by muscarinic antagonists, lead to massive increase of ACh release (Scali et al., 1995) that impinges on postsynaptic nAChRs, not blocked by muscarinic antagonists. Activation of postsynaptic nAChRs indirectly leads to activation of mTOR and formation of the mTORC1 complex that increases, through p70S6K activation, local protein synthesis that is necessary for IA LTM memory (Lana et al., 2013). A crosstalk between ERK and mTOR at different levels of the signalling flow is shown. Indeed, the mitogen-activated protein kinases have also been shown to regulate mTORC1. ERK was found to phosphorylate and inhibit the function of TSC2, albeit through different mechanisms and at different phosphorylation sites (Corradetti & Guan, 2006). For instance, a recent study (Tsokas, Ma, Iyengar, Landau, & Blitzer, 2007) showed an interesting interplay between ERK and mTOR pathways at CA3–CA1 synapses: ERK is required for the high frequency stimulation-induced activation of the mTOR pathway in the hippocampus. Further studies demonstrate a complex interplay among the cholinergic system, ERK and mTOR. For instance, mTOR is known to phosphorylate p70S6K at the site Thr389, while ERK is able to phosphorylate p70S6K at the site Thr421/Ser424 (Lafay-Chebassier et al., 2006). It was shown that the mAChRs agonist oxotremorine given IP induces phosphorylation of p70S6K at Thr389, which is not dependent upon activation of mTOR but possibly upon the ERK pathway activation (Deguil et al., 2008). Indeed, in a previous study, it was shown that mTOR could phosphorylate p70S6K at Thr421/Ser424, a specific site of ERK and inversely, ERK could phosphorylate p70S6K at Thr389 controlled by mTOR signalling (Lafay-Chebassier et al., 2006), making the story even more complex. It is also currently accepted that ERK and mTORC1 synergistically regulate Eukaryotic translation initiation factor 4E (eIF4E) and translation initiation in LTM and synaptic plasticity (Panja et al., 2009; Gal-Ben-Ari & Rosenblum, 2012).

It seems therefore that independent/concurrent/synergistic recruitment and activation of ERK and mTOR signalling cascades may be a conserved mechanism for the precise regulation of translation downstream of various neuromodulatory receptors.

5. Conclusions

The fascinating question on how and where memories are formed in our brain is the focus of intense investigations, and although a few answers are now available, we are still far from having a complete understanding of the process. In this review we summarized the present knowledge on the complex involvement of ACh, ERK and mTOR in the hippocampal mechanisms of IA memory. However, it must be kept in mind that other neurotransmitter systems and other signalling pathways are involved in the formation of IA memories. It should also be pointed out that the hippocampal structure is more complex than originally thought. Indeed, it has been demonstrated that the dorsal hippocampus has different functions from the ventral hippocampus in memory formation (Kheirbek et al., 2013). Furthermore, it is still a matter of investigation whether STM and LTM proceed in series, or in parallel. It has been described in rodent models that *de novo* protein synthesis is required to stabilize a STM into a LTM (Abel & Lattal, 2001), whereas others (Marti, Ramirez, Dos Reis, & Izquierdo, 2004) claim that STM and LTM are not processed by separate mechanisms. Nonetheless, the present view is that different molecular mechanisms may be needed to form STM and LTM (Lana et al., 2013), whereas some mechanisms are involved in both (Izquierdo et al., 1998a). Indeed, the demonstration that STM and LTM, acquired in a one-trial IA task, are independent phenomena, is given by the findings that pharmacological treatments block STM independently from LTM (Izquierdo et al., 1998a; Izquierdo, Medina, Vianna, Izquierdo, & Barros, 1999; Vianna, Izquierdo, Barros, Medina, & Izquierdo, 1999; Vianna et al., 2000; Izquierdo et al., 2002; Lana et al., 2013). Finally, with Tranel and Damasio (1995) we could conclude that "we have barely begun to unravel some of the mysteries of how our brains sub serve memory".

5. Legend to Figures

Figure 1. A schematic overview, based upon the literature and our own published work, of the intracellular pathways activated by mAChR and nAChR leading to STM and LTM IA encoding. In this scheme are also indicated the sites of action of the principal pharmacological tools used in the researches reviewed in this paper. Direct activation is represented by continuous arrows, indirect activation by dotted arrows. Black arrows indicate activation, T-shaped arrows indicate inhibition; open arrow indicates ACh release. IA: inhibitory avoidance STM: short term memory; LTM: long term memory.

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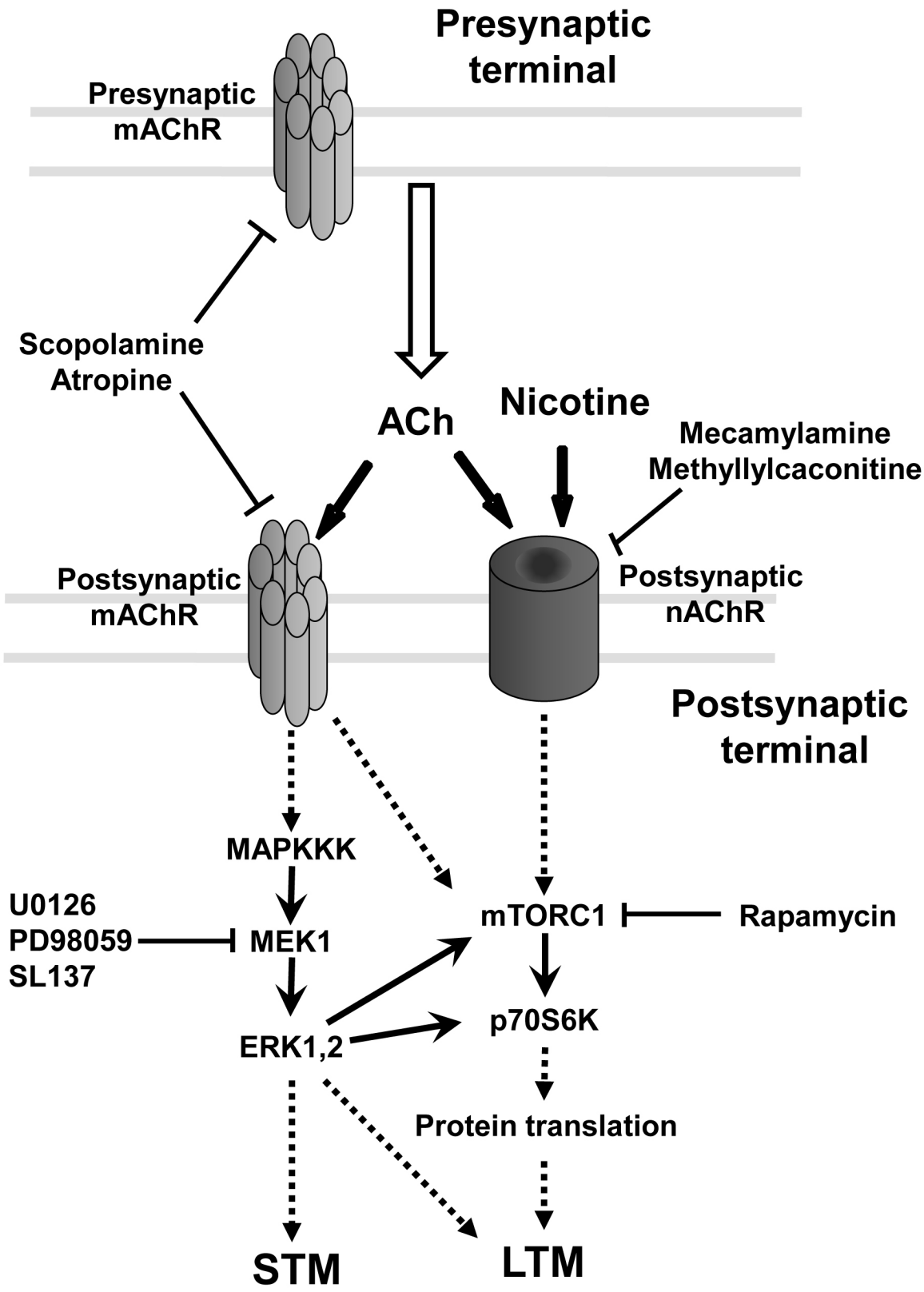
Figure 1


Presynaptic terminal
Presynaptic mAChR
Scopolamine
Atropine
ACh
Nicotine
Mecamylamine
Methyllylcaconitine
Postsynaptic mAChR
Postsynaptic nAChR
Postsynaptic terminal
MAPKKK
U0126
PD98059
SL137
MEK1
mTORC1
Rapamycin
p70S6K
ERK1,2
Protein translation
STM
LTM

Table

Table 1. *Effect of systemic or local administration of scopolamine on IA in rats and mice*

Inhibitory Avoidance	Species	Scopolamine Effective Doses	Route and time of administration	Effect	References
Step-through	Wistar Rats	8.0 mg, 16 mg	IP, posttraining	Impaired Recall at 24 h	(1)
Step-through	Wistar Rats	0.2 mg	IP, 30 min before training	Impaired Recall at 24 h	(2)
Step-through	Wistar Rats	1.0 mg	IP, 20 min before training	Impaired Recall at 24 h	(3)
Step-through	Wistar Rats	8.0 mg, 12 mg	IP, 5 min before training, Low footshock intensity IP, both 5 min before training and 5 min before recall, High footshok intensity	Impaired Recall Recall not changed (state-dependency)	(4)
Step-through	Wistar Rats	8.0 mg	IP, 5 min after training, Low footshock intensity	Impaired Recall at 24 h	(5)
Step-through	Mice (Std-ddY)	1.0 mg, 2.0 mg	IP, 30 min before training IP, immediately after training IP, 30 min before recall trial	Impaired Recall at 24 h Recall at 24not changed Recall at 24not changed	(6)
Step through	Wistar Rats	0.2 mg	SC, 30 min before training	Impaired recall (24h)	(7)
Step down	Wistar Rats	1.5 mg 1.5 mg 3 µg in 1µl	IP, 30 min before training IP, 30 min before recall Intrahippocampus (bilaterally)	Impaired recall (1 h) No effect (1 h) Impaired recall (1 h)	(8)
Step-down	Mice (Std-ddY)	0.5 mg, 1.0 mg 2.0 mg	IP, 30 min before training IP, immediately after training IP, 30 min before trecall trial	Impaired Recall at 24 h Recall at 24not changed Recall at 24not changed	(6)
Step-through	Mice (NMRI)	0.3 mg, 3.0 mg 30 mg	IP, 5 min before training IP, immediately after training IP, immediately after training	Impaired Recall at 24 h Recall at 24 h not changed Impaired Recall at 24 h	(9)
Step down	Wistar Rats	0.095 mg in 3 µl/side	Intrahippocampus (bilaterally)	Impaired Recall 5, 7 or 10 days but not 1 or 3 days after training	(10)
Step through	Wistar Rats	5-20 µg/rat	ICV, after training	Impaired Recall at 24 h	(11)

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